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Characterization of a Functional Hepatocyte Nuclear Factor 3 Binding Site in the Hepatitis B Virus Nucleocapsid Promoter[1]

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Mutational analysis of the hepatitis B virus (HBV) nucleocapsid promoter previously demonstrated that a regulatory sequence element (CpE) located between −72 and −56 modulated the level of transcription from this promoter in differentiated, but not dedifferentiated, hepatoma cell lines. Using gel retardation analysis, it was shown that the formation of a complex between the nucleocapsid CpE promoter sequence and the DNA-binding proteins present in the differentiated hepatoma cell line Huh7 was inhibited from forming in the presence of either the large surface antigen promoter hepatocyte nuclear factor 3 (HNF3) binding site or an HNF3β-specific antiserum. Purified recombinant HNF3α transcription factor was also shown to bind specifically to the CpE promoter sequence by gel retardation and DNase I footprinting analysis. In addition, DNase I footprinting analysis supported the suggestion that the nucleocapsid promoter region contains a second HNF3 binding site located between −112 and −86. The nucleocapsid promoter CpE regulatory element was shown to be a functional HNF3 binding site capable of mediating HNF3β-specific transcriptional transactivation in transient transfection analysis. These results suggest that the liver-enriched family of HNF3 transcription factors is involved in regulating the level of expression from the nucleocapsid promoter, in addition to the large surface antigen promoter, and is likely to be important in the coordinate regulation of HBV transcription during infection. © 1995 Academic Press, Inc.

INTRODUCTION

The hepatitis B virus (HBV) genome, as a consequence of its small size, appears to represent an appropriate system for examining coordinate liver-specific transcription from multiple promoters. The HBV genome is a partially double-stranded 3.2-kb DNA molecule which contains four promoters (Robinson *et al.*, 1974; Hruska *et al.*, 1977; Landers *et al.*, 1977; Sattler and Robinson, 1979; Siddiqui *et al.*, 1979; Charnay *et al.*, 1979; Sninsky *et al.*, 1979; Valenzuela *et al.*, 1979; Galibert *et al.*, 1979; Pasek *et al.*, 1979; Ono *et al.*, 1983; Raney *et al.*, 1990; Antonucci and Rutter, 1989; Ganem and Varmus, 1987; Raney and McLachlan, 1991). The enhancer I/X gene promoter controls the expression of a putative 0.7-kb RNA which is presumed to encode the X polypeptide (Araki *et al.*, 1989; Cheng and Moss, 1987; Kaneko and Miller, 1988; Guo *et al.*, 1991; Kim *et al.*, 1991; Wu *et al.*, 1991; Koike *et al.*, 1994). The major surface antigen promoter determines the level of the 2.1-kb RNA which is translated to generate the middle and major surface antigen polypeptides. The large surface antigen polypeptide is translated from the 2.4-kb RNA. The amount of the 2.4-kb RNA is determined by the large surface antigen promoter. The 3.5-kb RNA or pregenomic RNA is a multifunctional transcript which encodes the nucleocapsid and reverse transcriptase/DNA polymerase polypeptides (Cattaneo *et al.*, 1983, 1984; Yokosuka *et al.*, 1986; Su *et al.*, 1989; Kaneko and Miller, 1988; Ganem and Varmus, 1987; Raney and McLachlan, 1991). In addition, it is the pregenomic RNA which is reverse transcribed during the viral replication cycle and therefore represents the template for HBV genomic DNA biosynthesis (Will *et al.*, 1987; Ganem and Varmus, 1987; Raney and McLachlan, 1991).

During viral synthesis similar amounts of the 2.1- and 3.5-kb transcripts are observed, whereas a considerably lower amount of the 2.4-kb RNA appears to be present (Cattaneo *et al.*, 1983, 1984; Yokosuka *et al.*, 1986; Su *et al.*, 1989; Kaneko and Miller, 1988). This situation probably permits the correct abundances of the HBV polypeptides to be synthesized, consistent with the observation that the HBV viral envelope contains considerably less of the large surface antigen protein than the major surface antigen protein (Stibbe and Gerlich, 1982, 1983; Heermann *et al.*, 1984). More importantly, by comparison with the levels of expression in natural infection, it has been shown that higher levels of expression of the large surface antigen protein relative to the major surface antigen protein can inhibit the secretion of virus from the cell (Bruss and Ganem, 1991). This suggests that the relative levels of the 2.1- and 2.4-kb transcripts are regulated in a coordinated manner at the transcriptional level. Analysis of the relative strengths of the large surface antigen and major surface antigen promoters in differentiated hepatoma cell lines capable of replicating virus support this contention (Raney *et al.*, 1990).

The abundances of the 2.1- and 2.4-kb RNAs encoding

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the surface antigen polypeptides and the level of the 3.5-kb transcript which encodes the nucleocapsid polypeptide are probably coordinately regulated. This suggestion arises from the observation that the relative levels of these proteins determine how much of the mature nucleocapsids synthesized in the cell is recycled to the nucleus to maintain the level of the viral transcriptional template, the covalently closed circular HBV DNA, compared with the level of mature nucleocapsids which are enveloped by the surface antigen in the endoplasmic reticulum membrane and subsequently secreted from the cell (Tuttleman *et al.,* 1986; McLachlan, 1991; Lenhoff and Summers, 1994).

In addition to the coordinate expression of the HBV transcripts, it is apparent from the studies of transgenic mice containing the complete HBV genome that transcription from the viral genome is largely restricted to hepatocytes. In these cases, transcription of the viral DNA was largely restricted to the liver and to a lesser extent the kidney (Araki *et al.,* 1989; Farza *et al.,* 1988). This observation suggested that liver-enriched transcription factors are likely to be involved in the transcriptional regulation of the viral DNA. This suggestion has been supported by the observation that the liver-enriched transcription factors, C/EBP, HNF1, and HNF4, have been shown to influence the level of transcription from the HBV promoters (Chang *et al.,* 1989; Dikstein *et al.,* 1990b; Zhou and Yen, 1991; López-Cabrera *et al.,* 1991; Yuh and Ting, 1991; Raney *et al.,* 1991; Pei and Shih, 1990; Guo *et al.,* 1993; Garcia *et al.,* 1993; Raney *et al.,* 1994).

Previously it has been shown that HNF3 can increase the level of transcription from the large surface antigen promoter (unpublished observation). In this study, the role of the liver-enriched transcription factor, HNF3, in determining the level of transcription from the nucleocapsid promoter was examined. It was shown that the HNF3β present in the differentiated hepatoma cell line Huh7 specifically bound the previously identified nucleocapsid promoter regulatory sequence element (CpE) located between −72 and −56. This element was also shown to support transcriptional transactivation by exogenously expressed HNF3β. From these and previous results, it is apparent that HNF3 transcription factors may regulate the level of RNA synthesis directed by the nucleocapsid promoter, large surface antigen promoter (unpublished observation), and the X gene promoter/HBV enhancer I (Chen *et al.,* 1994). Therefore, the HNF3 transcription factor may be involved in the liver-specific and coordinate expression of the 0.7-, 2.4-, and 3.5-kb HBV transcripts during viral infection.

MATERIALS AND METHODS

Plasmid constructions

The steps in the cloning of the plasmid constructs used in the transfection experiments were performed by standard techniques (Sambrook *et al.,* 1989). The firefly luciferase (LUC) reporter gene in these constructions was derived from the plasmid, p19DLUC (Raney *et al.,* 1990). The plasmids pHBVTATALUC, CpA(2)TATALUC, CpB(3)TATALUC, CpE(3)TATALUC, CpF(3)TATALUC, HNF3(2)TATALUC, and HNF3(3)TATALUC were constructed by inserting synthetic double-stranded oligonucleotides into sites in the polylinker of p19DLUC. pHBVTATALUC was constructed by inserting a double-stranded oligonucleotide containing the large surface antigen promoter TATA-box element, produced by annealing the oligonucleotides CTATATTATATAAGAGAGAAGCT and TCTCTCTTATATAATATAGGTAC (spanning HBV coordinates 2773 to 2791), into the *Sac*I and *Kpn*I sites of p19DLUC in the same orientation as the TATA-box element occurs in the HBV genome (Raney *et al.,* 1994). CpA(2)TATALUC, CpB(3)TATALUC, CpE(3)TATALUC, CpF(3)TATALUC, HNF3(2)TATALUC, and HNF3(3)TATALUC were made by inserting two, three, or four copies (as indicated in the constructs designation) of the HBV CpA, CpB, CpE, CpF (Fig. 7) (Zhang *et al.,* 1993; Zhang and McLachlan, 1994), and PH (unpublished observation) double-stranded oligonucleotides into the unique *Sal*I site of pHBVTATALUC. The oligonucleotide pairs used to generate the CpA, CpB, CpE, CpF, and PH double-stranded oligonucleotides were TCGACACCGTGAACGCCCACCAAA and TCGATTTGGTGGGCGTTCACGGTG (oligo CpA, coordinates 1618 to 1638), TCGACTGGGAGGAGTT and TCGAAACTCCTCCCAG (oligo CpB, coordinates 1730 to 1743), TCGAGGGGGAGGAGAT and TCGAATCTCCTCCCCC (oligo CpC, coordinates 1744 to 1756), TCGAAGACTGTTTGTTTAAA and TCGATTTAAACAAACAGTCT (oligo CpE, coordinates 1713 to 1729), TCGATTAAAGGTCTTTGTACTAG and TCGACTAGTACAAAGACCTTTAA (oligo CpF, coordinates 1760 to 1778), and TCGACACTATTTACACACTCTATG and TCGACATAGAGTGTGTAAATAGTG (oligo PH, coordinates 2742 to 2763). The PH double-stranded oligonucleotide spans the large surface antigen HNF3 binding site (unpublished observation). The sequence of each construct was verified by dideoxynucleotide sequencing (Sanger *et al.,* 1977).

The pCMVHNF3α and pCMVHNF3β vectors express the HNF3α and HNF3β polypeptides from the HNF3α and HNF3β cDNAs, respectively, using the cytomegalovirus immediate–early promoter (Lai *et al.,* 1991; Pani *et al.,* 1992). The pCMV vector, which lacks a cDNA insert, was generated by digestion of pCMVHNF3α with *Eco*RI and subsequent ligation.

The pGEXHNF3α vector expresses a glutathione *S*-transferase (GST) fusion protein in *E. coli* DH5α which includes the complete HNF3α polypeptide sequence. The pGEXHNF3α vector was generated by cloning the 1.6-kbp HNF3α cDNA *Eco*RI fragment into the *Eco*RI site of the expression vector, pGEX-2T (Smith and Johnson, 1988). Expression and subsequent affinity purification of the GST-HNF3α fusion protein using glutathione–aga-

rose was performed as described (Smith and Johnson, 1988).

Cells and transfections

The human hepatoma cell lines Huh7 (Nakabayashi *et al.*, 1982) and HepG2.1 (Raney *et al.*, 1990) were grown in RPMI-1640 medium and 10% fetal bovine serum at 37° in 5% CO_2/air. The human cervical carcinoma cell line HeLa S3 (Puck *et al.*, 1956) was grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 mg/ml glucose and 10% calf serum at 37° in 5% CO_2/air. Transfections were performed as previously described (Graham and Van der Eb, 1973; Sorge *et al.*, 1984). The transfected DNA mixture comprised 15 μg of a LUC plasmid and 1.5 μg of pCMVβ, which served as an internal control for transfection efficiency. pCMVβ directs the expression of the *E. coli* β-galactosidase (β-gal) gene using the cytomegalovirus (CMV) immediate-early promoter (Clontech). The DNA mixture also included 1.5 μg of the HNF3β expression vector, pCMVHNF3β, or the control expression vector, pCMV. Cell extracts were prepared 40 to 48 hr after transfection and assayed for luciferase activity essentially as previously described (De Wet *et al.*, 1987). The extracts were measured for β-galactosidase activity using a Galacto-Light kit (Tropix, Inc.) as instructed by the manufacturer.

Nuclear and whole cell extracts, gel retardation analysis, and DNase I footprinting

Nuclear extracts were prepared from Huh7 and HepG2.1 cells essentially as described (Dignam *et al.*, 1983). All operations were performed at 0–4°. Cells were harvested from culture and centrifuged for 5 min at 2000 rpm in a Sorvall RT6000 centrifuge. Pelleted cells were washed once in 20 ml of 10 m*M* sodium phosphate (pH 6.8), 0.14 *M* NaCl, 1.5 m*M* $MgCl_2$ by suspension and recentrifugation for 5 min at 2000 rpm. The cell pellet was suspended in 5 vol of hypotonic buffer (10 m*M* Tris–hydrochloride (pH 7.9), 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol, 0.5 m*M* PMSF) and allowed to stand for 10 min. Cells were collected by centrifugation for 5 min at 2000 rpm, suspended in 2 vol of hypotonic buffer, and lysed by 10–15 strokes of an all-glass Dounce Homogenizer (B-type pestle). Cell lysis was more than 90%. The homogenate was centrifuged for 10 min at 3000 rpm in a Sorval SS34 rotor and the supernatant was carefully poured off to leave a loose nuclear pellet. This was recentrifuged for 20 min at 15,000 rpm in the SS34 rotor and the supernatant was decanted. The pellet was resuspended in 2.5 vol of nuclear extraction buffer (20 m*M* Tris–hydrochloride (pH 7.9), 25% (v/v) glycerol, 0.42 *M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 0.5 m*M* dithiothreitol, 0.5 m*M* PMSF), and the nuclei were lysed with 10 strokes of the homogenizer. The lysate was stirred gently for 30 min and then centrifuged for 30 min at 15,000 rpm in the SS34 rotor. The supernatant was decanted and dialyzed for 5 hr against 50 vol of 20 m*M* Tris–hydrochloride (pH 7.9), 20% (v/v) glycerol, 100 m*M* KCl, 0.2 m*M* EDTA, 0.5 m*M* dithiothreitol, 0.5 m*M* PMSF. The dialysate was clarified by centrifugation for 5 min at 14,000 rpm in a microcentrifuge and the supernatant was frozen in aliquots in liquid N_2 and stored at −80°.

Whole cell extracts were prepared from HeLa S3 cells by a rapid micropreparation technique as described (Andrews and Faller, 1991). HeLa S3 cells were transfected with 15 μg of the expression vectors encoding HNF3α or HNF3β 40 to 48 hr before preparation of the whole cell extracts.

Gel retardation analysis was performed essentially as described previously (Raney *et al.*, 1991). One nanogram of ^{32}P-labeled double-stranded CpE oligonucleotide (see Plasmid Constructions) was incubated with 9 μg extract prior to 4% polyacrylamide gel electrophoresis and autoradiography. When gel retardation competition analysis was performed, the extract was preincubated with 1 μg of cold double-stranded competitor oligonucleotide for 15 min prior to the addition of the ^{32}P-labeled double-stranded CpE oligonucleotide. Gel retardation analysis using antibodies was performed essentially as described previously (Le *et al.*, 1994). The extract was incubated with 2 μl of antiserum specific for the HNF3α, HNF3β, or HNF3γ polypeptide (Lai *et al.*, 1991) for 1 hr prior to the addition of the ^{32}P-labeled double-stranded CpE oligonucleotide.

The DNase I footprinting assay was performed as described previously (Raney *et al.*, 1989, 1992; Briggs *et al.*, 1986). The reactions contained 1 to 5 ng of end-labeled DNA fragment in a 100 μl reaction mixture containing 10 m*M* Tris–hydrochloride (pH 7.9), 50 m*M* KCl, 0.5 m*M* EDTA, 1 m*M* DTT, 0.5 m*M* PMSF, 10% (vol/vol) glycerol, and purified recombinant GST or GST-HNFα polypeptide. Binding was carried out for 15 min at 0° and then for 2 min at room temperature, after which 100 μl of 5 m*M* $CaCl_2$, 10 m*M* $MgCl_2$, containing DNase I, was added at room temperature. The reaction mixture was incubated for 2 min, after which the reaction was stopped by the addition of 200 μl of 1% (wt/vol) sodium dodecyl sulfate (SDS), 20 m*M* EDTA, 200 m*M* NaCl, containing 125 μg of tRNA per ml. The mixture was precipitated with ethanol and analyzed by 6% urea–acrylamide sequencing gel electrophoresis and autoradiography.

RESULTS

A DNA-binding protein from the differentiated hepatoma cell line Huh7 which interacts with the CpE regulatory sequence element of the HBV nucleocapsid promoter is HNF3β

The influence of the liver-enriched transcription factor HNF3 on the transcriptional activities of the four HBV promoters was previously measured in the dedifferentiated hepatoma cell line HepG2.1 using transient transfection analysis (unpublished observation). The tran-

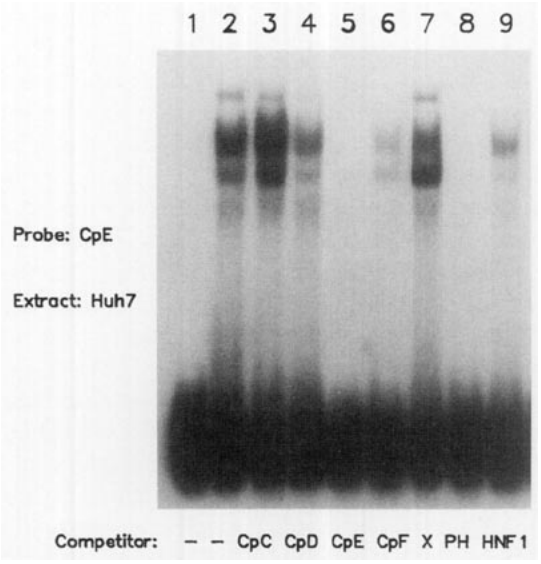


FIG. 1. Gel retardation and complex inhibition analysis of the HBV nucleocapsid promoter CpE regulatory sequence element. The ^{32}P-labeled, double-stranded oligonucleotide, CpE (Fig. 7), and Huh7 nuclear extract were used for this analysis. Extract was omitted from lane 1. Unlabeled, double-stranded oligonucleotides were used as competitor DNAs to demonstrate the specificity of the observed complexes. CpC (lane 3), nucleocapsid promoter Sp1 binding site (Zhang *et al.*, 1993); CpD (lane 4), nucleocapsid promoter control oligonucleotide (Zhang *et al.*, 1993); CpE (lane 5), nucleocapsid promoter liver-specific factor binding site with homology to the HNF3 recognition sequence (Zhang and McLachlan, 1994); CpF (lane 6), nucleocapsid promoter liver-specific factor binding site (Zhang and McLachlan, 1994); X (lane 7), X gene promoter region oligonucleotide (Zhang and McLachlan, 1994); PH (lane 8), large surface antigen promoter HNF3 binding site (unpublished observation); HNF1 (lane 9), large surface antigen promoter HNF1 binding site (Raney *et al.*, 1991).

scriptional activity from the large surface antigen promoter was increased by the expression of the HNF3β polypeptide approximately 90-fold, whereas the activity of the nucleocapsid promoter was increased approximately 3-fold. The level of transcription from the other two HBV promoters was not influenced by HNF3. This suggested that as in the case of the large surface antigen promoter, the nucleocapsid promoter might also contain HNF3 binding sites. This was supported by the observation that the CpE regulatory element from the nucleocapsid promoter was able to inhibit the complex formation between the large surface antigen HNF3 binding site and HNF3 polypeptides (unpublished observation).

The possibility that the CpE regulatory element from the nucleocapsid promoter might bind HNF3 related transcription factors was examined by gel retardation analysis (Figs. 1–4). The complexes formed between the CpE regulatory element and factors in Huh7 extracts were examined and one major and two minor complexes were observed (Fig. 1, lane 2). To determine the specificity of the interaction between the factors and the CpE binding site oligonucleotide, several unlabeled, double-stranded oligonucleotides were used as competitors in the gel retardation analysis. Unlabeled, double-stranded oligonucleotides representing a nucleocapsid promoter Sp1 binding site (CpC), a control oligonucleotide (CpD), and an X gene promoter/enhancer I region oligonucleotide (X) failed to compete for the factor(s) which form the major complex with the CpE oligonucleotide. An unknown liver factor binding site (CpF) and the large surface antigen promoter HNF1 binding site (HNF1) partially competed for the factor(s) forming the major complex with the CpE oligonucleotide. These oligonucleotides are identical to the HNF3 consensus sequence at 8 and 10 of 12 positions, respectively. The partial competition observed with these oligonucleotides probably reflects the ability of these sequences to bind HNF3-related factors when present at relatively high concentrations. This has previously been reported for the CpF oligonucleotide (Zhang and McLachlan, 1994). The unlabeled nucleocapsid promoter CpE oligonucleotide and the large surface antigen promoter HNF3 binding site PH oligonucleotide efficiently competed with the ^{32}P-labeled CpE oligonucleotide for binding the factors in the Huh7 extract. The nucleocapsid promoter CpE oligonucleotide and the large surface antigen promoter HNF3 binding site PH oligonucleotide contain 11 and 10 nucleotides, respectively, which are identical to the 12-nucleotide HNF3 binding site consensus sequence (Fig. 7) (Overdier *et al.*, 1994). These results suggest that the DNA-binding proteins which form complexes with the CpE regulatory

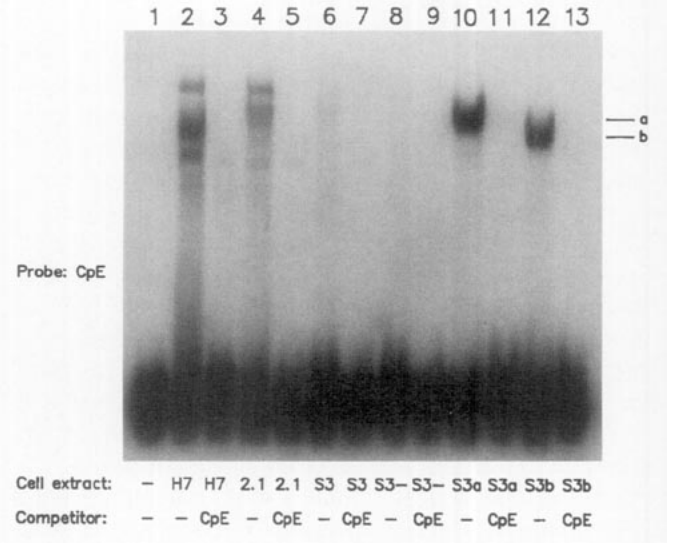


FIG. 2. Gel retardation and complex inhibition analysis of the HBV nucleocapsid promoter CpE regulatory sequence element DNA–protein complexes formed with different extracts. The ^{32}P-labeled, double-stranded oligonucleotide, CpE (Fig. 7), was used for this analysis. Analysis of the complexes formed with the DNA-binding proteins present in untransfected Huh7 nuclear extract (H7, lanes 2 and 3), HepG2.1 nuclear extract (2.1, lanes 4 and 5), HeLa S3 whole cell extract (S3, lanes 6 and 7), whole cell extract from HeLa S3 cells transfected with pCMV (S3-, lanes 8 and 9), whole cell extract from HeLa S3 cells transfected with pCMVHNF3α (S3a, lanes 10 and 11), and whole cell extract from HeLa S3 cells transfected with pCMVHNF3β (S3b, lanes 12 and 13). Extract was omitted from lane 1. The unlabeled, competitor CpE oligonucleotide was included in lanes 3, 5, 7, 9, 11, and 13.

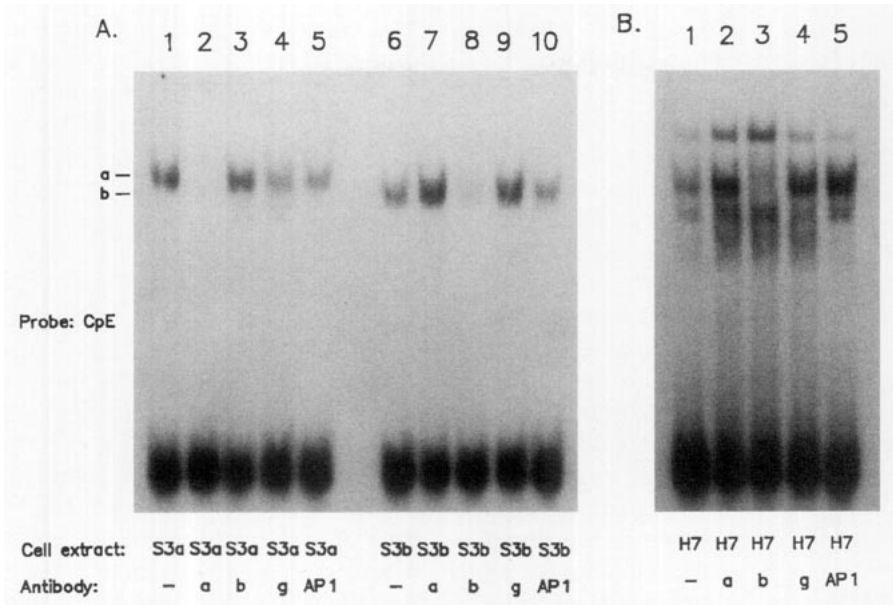


FIG. 3. Analysis of the inhibition of complex formation by HNF3-specific antisera. The ^{32}P-labeled, double-stranded oligonucleotide, CpE (Fig. 7), was used for this analysis. (A) Analysis of the complexes formed with the DNA-binding proteins present in HeLa S3 cells transfected with pCMVHNF3α (S3a, lanes 1–5) and HeLa S3 cells transfected with pCMVHNF3β (S3b, lanes 6–10). Anti-HNF3α antiserum (a, lanes 2 and 7), anti-HNF3β antiserum (b, lanes 3 and 8), anti-HNF3γ antiserum (g, lanes 4 and 9), and anti-AP1 antibodies (AP1, lanes 5 and 10) were included as indicated. (B) Analysis of the complexes formed with the DNA-binding proteins present in Huh7 nuclear extracts (H7). Anti-HNF3α antiserum (a, lane 2), anti-HNF3β antiserum (b, lane 3), anti-HNF3γ antiserum (g, lane 4), and anti-AP1 antibodies (AP1, lane 5) were included as indicated.

sequence are likely to be HNF3-related transcription factors.

Three different cell line extracts were compared in the gel retardation analysis to examine the cell line specificity of the factors (Fig. 2). As reported previously, the extracts derived from the differentiated hepatoma cell line Huh7 and the dedifferentiated hepatoma cell line HepG2.1 formed different complexes with the CpE regulatory sequence (Zhang and McLachlan, 1994). Complexes were not observed when the cervical carcinoma cell line HeLa S3 extracts were analyzed (Zhang and McLachlan, 1994). The gel retardation complexes were

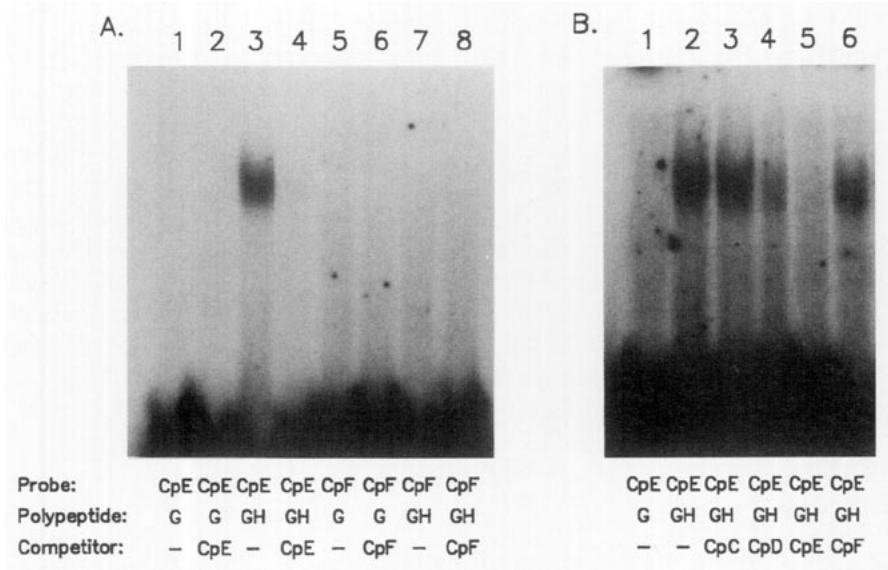


FIG. 4. Gel retardation and complex inhibition analysis of the HBV nucleocapsid promoter CpE regulatory sequence element. (A) The ^{32}P-labeled, double-stranded oligonucleotides, CpE (lanes 1–4) and CpF (lanes 5–8) (Fig. 7), and purified recombinant GST (G, lanes 1, 2, 5, and 6) and GST-HNF3α (GH, lanes 3, 4, 7, and 8) were used for this analysis. The unlabeled, double-stranded oligonucleotides CpE (lanes 2 and 4) and CpF (lanes 6 and 8) were used as competitor DNAs to demonstrate the specificity of the observed complex. (B) The ^{32}P-labeled, double-stranded oligonucleotide, CpE (Fig. 7), and purified recombinant GST (G, lane 1) and GST-HNF3α (GH; lanes 2–6) were used for this analysis. Unlabeled, double-stranded oligonucleotides were used as competitor DNAs to demonstrate the specificity of the observed complex. CpC (lane 3), CpD (lane 4), CpE (lane 5), and CpF (lane 6) are as described in Fig. 1.

also compared with the complexes formed by the exogenous expression of HNF3 polypeptides. The major gel retardation complex formed with extracts from the differentiated hepatoma cell line Huh7 appeared to have similar migration properties to the exogenously expressed HNF3β in HeLa S3 cells. These results suggest that the Huh7 cells express a factor which may be the same or similar to the HNF3β polypeptide, whereas the HepG2.1 cells do not appear to produce the HNF3β transcription factor. This is consistent with the liver-enriched distribution of the HNF3 polypeptides (Lai *et al.*, 1990, 1991; Xanthopoulos *et al.*, 1991). The mobility of the exogenously expressed HNF3α polypeptide-DNA complex is slightly less than that of the HNF3β polypeptide, consistent with previously reported gel retardation analyses (Lai *et al.*, 1990, 1991). The major gel retardation complex formed with extracts from the dedifferentiated hepatoma cell line HepG2.1 appeared to have migration properties similar to those of the exogenously expressed HNF3α in HeLa S3 cells. However, additional analysis (see below) indicates that the majority of the DNA–protein complexes formed between the CpE oligonucleotide and the HepG2.1 extract did not contain the HNF3α polypeptide.

Gel retardation inhibition analysis was also performed using appropriate antisera to characterize further the nature of the gel retardation complexes (Fig. 3). HeLa S3 extract containing exogenously expressed HNF3α or HNF3β polypeptide formed complexes which could be inhibited from forming by the addition of the corresponding antiserum against HNF3α or HNF3β (Lai *et al.*, 1991). The formation of the major complex between the Huh7 extract and the CpE oligonucleotide was inhibited only by the HNF3β antiserum, not by the HNF3α antiserum, the HNF3γ antiserum, or the unrelated control polyclonal antipeptide c-jun/AP1 antibody. This is consistent with the observation that the complex which was inhibited from forming by the HNF3β antiserum was the Huh7 DNA–protein complex which comigrated with the complex formed between the exogenously expressed HNF3β in the HeLa S3 extract and the CpE oligonucleotide. The results of the gel retardation inhibition analysis and the migration properties of a complex formed with the Huh7 extract suggest that one of the factors present in Huh7 cells which binds the nucleocapsid promoter CpE binding site is HNF3β. Similar experiments failed to reveal easily detectable levels of HNF3α, HNF3β, or HNFγ polypeptides in the complexes formed between the CpE oligonucleotide and the DNA binding proteins in the HepG2.1 extract (unpublished observation).

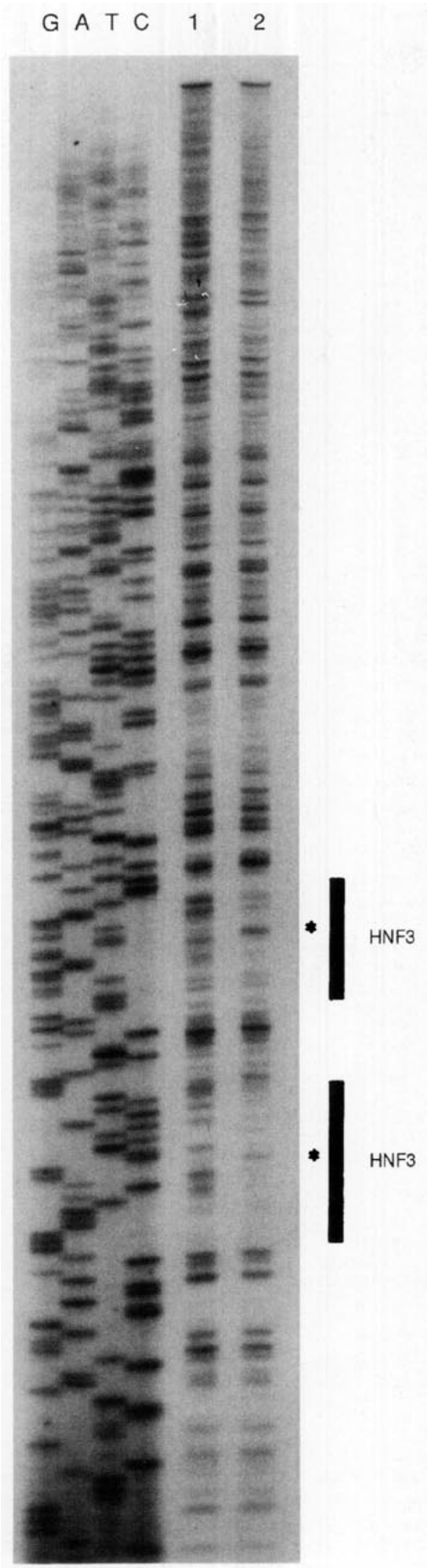
Identification of the HNF3 transcription factor binding sites in the nucleocapsid promoter

Previous analysis of the nucleocapsid promoter had indicated that some of the DNA binding proteins which interacted with the CpE and CpF regulatory elements (Fig. 7) might be related (Zhang and McLachlan, 1994). To examine the possibility that the HNF3 transcription factors might interact with both of these regulatory elements in the nucleocapsid promoter, the interactions between purified recombinant GST-HNF3α polypeptide and these elements were analyzed (Fig. 4). Gel retardation analysis demonstrated that the CpE regulatory element specifically bound the purified recombinant GST-HNF3α polypeptide (Fig. 4A, lanes 1–4), whereas the CpF regulatory element failed to bind the GST-HNF3α polypeptide (Fig. 4A, lanes 5–8). In addition, the specificity of the complex formed between the CpE regulatory sequence element and the GST-HNF3α polypeptide was examined further by demonstrating that only the CpE oligonucleotide, and not the CpC, CpD, or CpF oligonucleotides, inhibited complex formation (Fig. 4B).

The regulatory sequence elements of the nucleocapsid promoter that interact with the GST-HNF3α polypeptide were characterized further by DNase I footprinting analysis (Fig. 5). The expected HNF3 transcription factor DNase I footprint over the region of the CpE regulatory sequence element was observed. This DNase I footprint, HNF3 site 2, spans the promoter region from −71 to −45 and includes the sequence from −70 to −59, which is identical to the HNF3 consensus sequence at 11 of 12 nucleotides (Fig. 7) (Overdier *et al.*, 1994). Also as expected from the gel retardation results (Fig. 4), a DNase I footprint was not observed in the region of the CpF regulatory sequence. However, in addition to the HNF3 transcription factor DNase I footprint over the CpE regulatory sequence, a second DNase I footprint was observed just upstream of the HNF3 site 2 (Fig. 5). This observation suggests that a second HNF3 site, HNF3 site 1, might be located between −112 and −86 in the nucleocapsid promoter. Within the region of the DNase I protection, the sequence from −105 to −94 is identical to the HNF3 consensus sequence at 8 of 12 nucleotides (Fig. 7) (Overdier *et al.*, 1994). Further analysis will be required to confirm definitively the properties of this putative HNF3 recognition sequence. However, the presence of GST-HNF3α polypeptide-induced DNase I hypersensitive sites characteristic of HNF3 transcription factor footprints (Liu *et al.*, 1991; McPherson *et al.*, 1993) within both DNase I protected regions supports the suggestion that the nucleocapsid promoter has two HNF3 binding sites (Fig. 5).

Transactivation of transcription by the HNF3β transcription factor mediated through the CpE synthetic nucleocapsid HNF3 binding site

Mutational analysis of the nucleocapsid promoter has demonstrated that the CpE regulatory sequence modulates the level of transcription from this promoter only in differentiated hepatoma cell lines (Zhang and McLachlan, 1994). This suggested that a liver-enriched transcription factor might mediate the observed transcriptional effects on the nucleocapsid promoter. The liver-enriched



HNF3 transcription factor has been shown to transactivate transcription weakly from the nucleocapsid promoter (unpublished observation). The approximately threefold induction which was observed was not sufficient to map directly the HNF3-responsive element(s) regulating the level of transcription from the nucleocapsid promoter (unpublished observation). Consequently, several of the previously identified regulatory elements of the nucleocapsid promoter (Fig. 7) (Zhang *et al.*, 1993; Zhang and McLachlan, 1994) were examined in the context of a minimal promoter to determine if they could mediate HNF3-specific transcriptional transactivation.

The nucleocapsid promoter regulatory sequence elements (Fig. 7) were examined in the context of a minimal TATA-box element located upstream of the luciferase open reading frame (ORF) (Fig. 6). These synthetic promoter constructs were tested for their transcriptional activities in the HepG2.1 cell line in the absence (−HNF3β) or presence (+HNF3β) of the HNF3β expression vector (Fig. 6). The activities were shown relative to the activity of the synthetic construct containing three copies of the CpE oligonucleotide (construct CpE(3)TATALUC) in the presence of exogenously expressed HNF3β (+HNF3β). The level of transcription from this promoter was increased at least 50-fold in the presence of the HNF3β expression vector. Control constructs, HNF3(2)TATALUC and HNF3(3)TATALUC, containing HNF3 binding sites from the large surface antigen promoter showed similar levels of transcriptional activities to the CpE(3)TATALUC construct, both in the presence and absence of exogenously expressed HNF3β. This indicates that the nucleocapsid promoter CpE regulatory element represents a functional HNF3 binding site, at least in the context of a minimal promoter. In contrast, the unrelated synthetic oligonucleotide constructs, CpA(2)TATALUC and CpB(3)TATALUC, containing nucleocapsid promoter Sp1 transcription factor binding sites (Fig. 7) cloned into pHBVTATALUC did not show transcriptional transactivation by exogenously expressed HNF3β (Fig. 6). The synthetic oligonucleotide construct, CpF(3)TATALUC, containing the CpF nucleocapsid promoter regulatory element (Fig. 7) cloned into pHBVTATALUC also did not show transcriptional transactivation by exogenously expressed HNF3β (Fig. 6). Therefore, of the regulatory elements examined, only the CpE regulatory element appears to be a functional site which can mediate specific transcriptional transactivation by HNF3β.

FIG. 5. DNase I footprinting analysis of the long (or minus) strand of the HBV nucleocapsid promoter. A 1119-nucleotide HBV DNA fragment from −1099 to +20 was 5′-end labeled at +20 (lanes 1 and 2) and incubated in the presence of purified recombinant GST (lane 1) or GST-HNF3α (lane 2). The top and bottom HNF3 footprints are designated HNF3 sites 1 and 2, respectively (Fig. 7). Asterisks indicate the DNase I hypersensitive sites within the HNF3 footprints. An unrelated DNA sequence (GATC) is adjacent to lane 1 and was used as a size standard to locate the HNF3 binding sites.

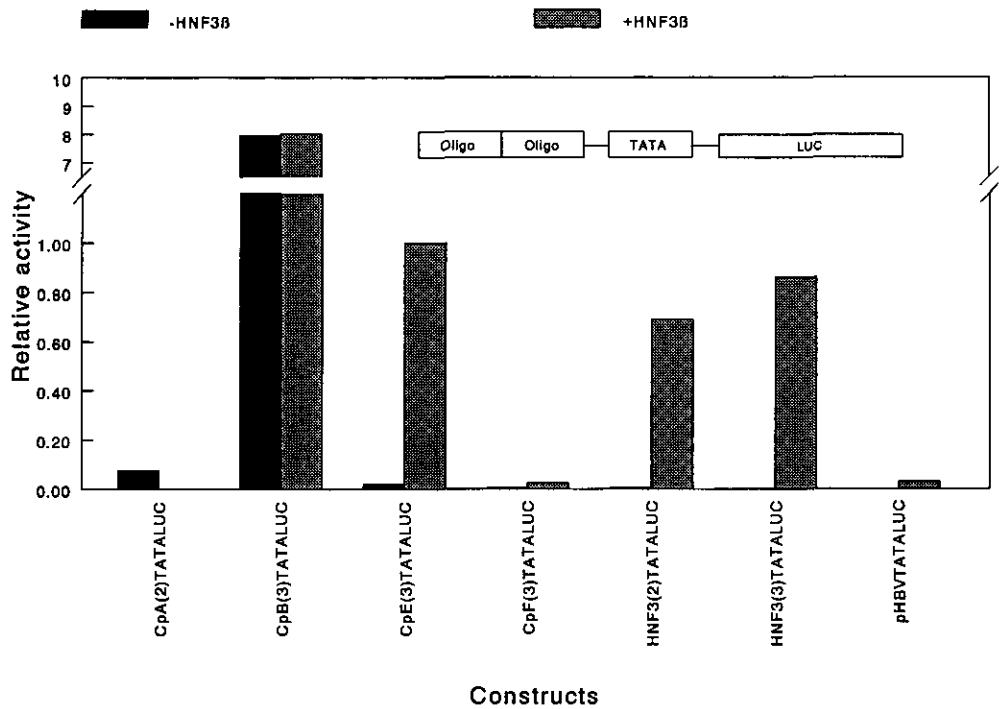


FIG. 6. Functional analysis of the HBV nucleocapsid promoter CpE regulatory sequence element. The constructs examined contain the CpA, CpB, CpE, CpF (Fig. 7), and the large surface antigen promoter HNF3 double-stranded oligonucleotide, PH (unpublished data), cloned into the minimal promoter construct, pHBVTATALUC (see insert) (Raney *et al.*, 1994). The constructs CpA(2)TATALUC, CpB(3)TATALUC, CpE(3)TATALUC, CpF(3)TATALUC, HNF3(2)TATALUC, and HNF3(3)TATALUC contain two or three copies of the indicated double-stranded oligonucleotide cloned into pHBVTATALUC. The relative activities of the constructs in the absence (−HNF3β) or presence (+HNF3β) of the expression vector, pCMVHNF3β, were examined in HepG2.1 cells. The transcriptional activities are reported relative to the CpE(3)TATALUC construct in the presence of exogenously expressed HNF3β which is designated as having a relative activity of 1.0. The internal control used to correct for transfection efficiencies was pCMVβ.

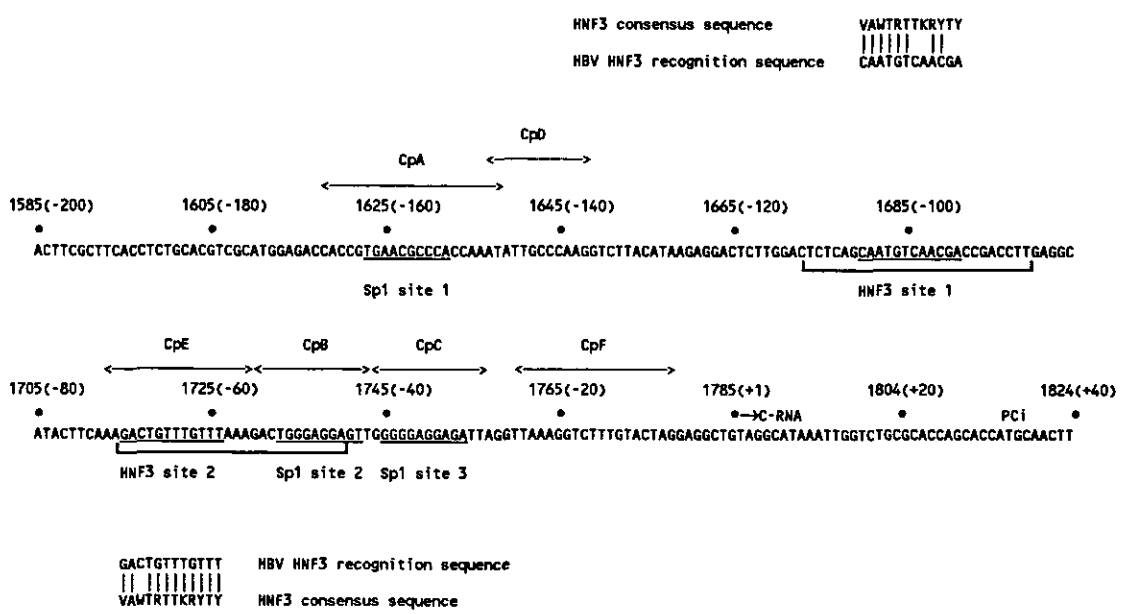


FIG. 7. Nucleotide sequence of the HBV nucleocapsid promoter region (subtype *ayw*) (Galibert *et al.*, 1979). The nucleotide coordinates are derived from the GenBank database and their positions relative to the transcription initiation site (+1; nucleotide coordinate 1785) are indicated (Yaginuma *et al.*, 1987; Sells *et al.*, 1988; Honigwachs *et al.*, 1989; Waisman *et al.*, 1990; Hu and Siddiqui, 1991). The underlined sequences represent the regions of homology to the Sp1 and HNF3 recognition sequences. The sequences of the CpA, CpB, CpC, CpD, CpE, and CpF oligonucleotides are indicated. The brackets under the nucleotide sequence span the regions protected from DNase I digestion by purified recombinant GST-HNF3α (Fig. 5). The HBV nucleocapsid promoter HNF3 recognition sequences are shown, and the nucleotides homologous to the HNF3 consensus sequence (Overdier *et al.*, 1994) are indicated (K = G/T; R = A/G; V = A/C/G; W = A/T; Y = C/T).

DISCUSSION

The detailed analysis of mutated nucleocapsid promoters identified the CpE regulatory sequence element which was functionally important for promoter activity only in differentiated hepatoma cell lines (Zhang and McLachlan, 1994). This regulatory element was located between −72 and −56 relative to a transcription start site (Fig. 7) (Yaginuma *et al.*, 1987; Sells *et al.*, 1988; Honigwachs *et al.*, 1989; Waisman *et al.*, 1990; Hu and Siddiqui, 1991). The DNA binding proteins from differentiated hepatoma cell lines which formed complexes with the CpE regulatory element appeared to be different from those identified in dedifferentiated hepatoma cell lines (Zhang and McLachlan, 1994). These observations correlated promoter activity with the formation of specific DNA–protein complexes. However, the transcription factor(s) mediating these effects was unknown. In this study, gel retardation analysis demonstrated that either an unrelated HNF3 competitor oligonucleotide derived from the large surface antigen promoter sequence (unpublished observation) or an antiserum directed against the HNF3β transcription factor could specifically inhibit the formation of a complex formed between Huh7 extract and the CpE regulatory sequence element (Figs. 1–3). This indicated that the CpE regulatory sequence is an HNF3 recognition site. In addition, it suggests a possible explanation for the differences in nucleocapsid promoter regulatory sequence elements identified in the Huh7 and HepG2.1 cell lines (Zhang and McLachlan, 1994). In Huh7 cells, HNF3β activates transcription from the CpE sequence revealing the importance of this regulatory element. HepG2.1 cells lack HNF3β and consequently the CpE sequence cannot modulate nucleocapsid promoter activity in these cells. Transient transfection analysis demonstrated that the CpE regulatory sequence element was functional and could specifically mediate transcriptional transactivation by HNF3β (Fig. 6). DNase I footprinting confirmed the CpE regulatory element specifically bound the HNF3 transcription factor and also revealed a second HNF3 binding site, HNF3 site 1, upstream of the CpE regulatory sequence, HNF3 site 2 (Figs. 5 and 7). HNF3-binding site 1 is located in a region of the nucleocapsid promoter which had previously been shown to modulate the level of transcription from this promoter between two- and fourfold in differentiated hepatoma cell lines but less than twofold in the dedifferentiated hepatoma cell line HepG2.1 and the HeLa S3 cell line (Zhang *et al.*, 1992). These observations are consistent with the possibility that HNF3β might be mediating these changes in transcriptional activity from the nucleocapsid promoter in the differentiated hepatoma cell lines by binding to HNF3 site 1.

It is apparent that only one of the complexes formed between the CpE regulatory sequence and Huh7 extracts has been demonstrated to involve HNF3β. The remaining complexes formed with Huh7 and HepG2.1 extracts presumably arise from additional HNF3 binding-site-specific DNA–protein interactions. Based on the inhibition of complex formation using specific antiserum, it appears that Huh7 extracts lack readily detectable HNF3α and HNF3γ (Fig. 3) and HepG2.1 extracts appear to lack HNF3α, HNF3β and HNF3γ (unpublished observation). This suggests that the unidentified complexes formed with the CpE regulatory element may include additional members of the HNF3/*forked head* family of transcription factors (Lai *et al.*, 1991; Clevidence *et al.*, 1993; Kaestner *et al.*, 1993). In the case of HepG2.1 cells, any members of the HNF3/*forked head* family of transcription factors which might interact with the CpE regulatory element presumably have little effect on the level of transcription from the nucleocapsid promoter (Zhang and McLachlan, 1994).

It is apparent from the analysis of transgenic mice and transfection experiments in cell culture that transcription of the HBV genome occurs preferentially in cells of hepatic origin (Araki *et al.*, 1989; Farza *et al.*, 1988; Raney *et al.*, 1990, 1991, 1994; Jameel and Siddiqui, 1986; Karpen *et al.*, 1988; Antonucci and Rutter, 1989; Honigwachs *et al.*, 1989; Yee, 1989; Chang *et al.*, 1989; Patel *et al.*, 1989; Shaul and Ben Levy, 1987; Shaul *et al.*, 1985; Tognoni *et al.*, 1985; Yuh and Ting, 1993; Zhang and McLachlan, 1994). The reason for this is becoming apparent as the transcription factors which interact with the viral promoter sequences are identified. Several liver-enriched transcription factors, including C/EBP, HNF1, HNF3, and HNF4, appear to have important roles in determining the level of transcription from the HBV promoters (Raney *et al.*, 1990, 1991, 1994; Chang *et al.*, 1989; Nakao *et al.*, 1989; López-Cabrera *et al.*, 1990, 1991; Dikstein *et al.*, 1990b; Zhou and Yen, 1991; Yuh and Ting, 1991; Pei and Shih, 1990; Guo *et al.*, 1993; Garcia *et al.*, 1993; Chen *et al.*, 1994). In addition, transcription factors which appear to have a more ubiquitous cellular distribution, including AP1, EF-C/RFX, NF1, and Sp1, also appear to be involved in determining the level of expression from the HBV promoters (Patel *et al.*, 1989; Faktor *et al.*, 1990; Shaul *et al.*, 1986; Dikstein *et al.*, 1990a; Ben-Levy *et al.*, 1989; Raney *et al.*, 1992; Zhang *et al.*, 1993; Garcia *et al.*, 1993; Siegrist *et al.*, 1993; Reith *et al.*, 1994; Ostapchuk *et al.*, 1989). It is presumably the combined action of these and as yet unidentified transcription factors which determine the level of expression of the HBV transcripts during viral infection (Garcia *et al.*, 1993). The levels of the various transcripts must be controlled so that the viral polypeptides are made in the correct abundances for viral biogenesis (Raney and McLachlan, 1991). One possible mechanism which may have a role in coordinately modulating the appropriate levels of multiple transcripts simultaneously would employ a specific transcription factor to control the level of transcription from more than one HBV promoter. This has been reported for C/EBP and HNF4 which modulate the activity of the enhancer I/X gene promoter and the nucleocapsid promoter

(Patel *et al.*, 1989; López-Cabrera *et al.*, 1990, 1991; Dikstein *et al.*, 1990b; Pei and Shih, 1990; Guo *et al.*, 1993; Garcia *et al.*, 1993). The NF1 and Sp1 transcription factors regulate the activities of the major surface antigen and enhancer I/X gene promoters (Shaul *et al.*, 1986; Ben-Levy *et al.*, 1989) and the major surface antigen and nucleocapsid promoters (Raney *et al.*, 1992; Zhang *et al.*, 1993), respectively. In the present study, it is apparent that the HNF3 transcription factor positively regulates the nucleocapsid promoter activity in addition to increasing the transcription from the large surface antigen promoter (unpublished observation) and modulating enhancer I/X gene promoter activities (Chen *et al.*, 1994). The combinatorial action of the transcription factors which regulate more than one HBV promoter probably permits modulation of HBV transcription so that the viral transcripts are always made in abundances appropriate for viral biogenesis independently of changes in the absolute level of transcription factors within the hepatocyte.

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